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Maternal Inhibition of Hatching at High Population Densities in *Tigriopus japonicus* (Copepoda, Crustacea)

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Abstract. A new mode of maternal protection is described for organisms that maintain contact with their developing embryos until hatching. Females of the copepod *Tigriopus japonicus* inhibit hatching of mature embryos (nauplii) from eggs they carry. Inhibition occurs at high population densities, or in the medium from crowded cultures. In contrast, when the mothers are killed or detached from their mature egg-sacs, all nauplii hatch within an hour, even in media from high-density cultures. A structure probably serving as an "umbilical cord" for transmission of the inhibitory message was demonstrated using electron microscopy.

Introduction

Population regulation by density-dependent mechanisms has been established for a wide variety of organisms (Peters and Barbosa, 1977; Stebbing and Heath, 1984), including copepods (Hicks and Coull, 1983)—crustacea that are ubiquitous in aquatic habitats. Progress in cultivation of harpacticoid copepods has resulted in high-density cultures for research and possibly aquaculture (Rothbard, 1976; Kahan, 1979, 1981; Kahan and Azoury, 1981; Chandler, 1986). In *Tigriopus japonicus* Mori (Mori, 1938; Ito, 1970)—a marine harpacticoid widely used in hatcheries in the Far East (Kuronuma and Fukusho, 1984) and reared in our laboratory—the percentage of females carrying mature egg-sacs increased in dense populations. This phenomenon, occurring under continuous or diurnal illumination, may signify a delay in hatching of mature embryos. Maternally induced arrest of embryonic development occurs in various organisms that lay eggs and release them (Gilbert, 1974; Clegg and Conte, 1980; Marcus, 1982; Yamashita and Hasegawa, 1985); it also occurs in certain mammals (Renfree, 1978). The present study is the first to describe delay in hatching of mature embryos from an egg-sac carried by the mother—a delay mediated by the living mother. Furthermore, a structure that resembles the "hooklets" described in another harpacticoid copepod (Fahrenbach, 1962) and that may serve as an "umbilical cord" transmitting the inhibitory message is shown.

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Materials and Methods

Tigriopus japonicus, (obtained from Ms. Huei-Mei Su Tseng, Tungkang Marine Laboratories, Taiwan) was cultivated in artificial seawater, salinity 35‰, prepared from Instant Ocean salts (Aquarium Systems, Mento, Ohio) in covered, rectangular, glass aquaria (30 × 16 × 20 cm) each containing 6 liters of medium. The cultures were maintained in a room at 20 ± 2°C with continuous lighting, hence permitting development of algae, and fed ad libitum on wheat germ. Like most harpacticoids, females of *T. japonicus* lay eggs in consecutive batches *i.e.*, egg-sacs, each attached to the abdomen. The emerging eggs (dark green) become orange-red during maturation. Two to three days pass between laying and the hatching of nauplii. In each experiment, performed under similar conditions, females carrying mature egg-sacs taken from the same high-density culture (5[illegible] per ml) were placed in conditioned or control seawater at the densities specified, with a flake (about 0.5 × 1 mm) of wheat germ, in plastic cells 1.6 cm diameter (Multidish, Nunc, Denmark). Conditioned medium was obtained from the high-density cultures by removing the copepods and filtering the medium through filter paper. Filtered fresh

medium, in which no organisms had been cultivated, was the control medium.

Hatching was observed under a dissecting microscope. All nauplii from the same egg-sac hatched almost simultaneously within a few minutes.

Effect of density on hatching

Because inhibition of hatching seemed likely in high-density cultures, the influence of population density was investigated first. Females carrying mature egg-sacs were taken from a dense culture and placed in a conditioned medium at varying densities: 40, 20, 10, 5, and 1 egg-carrying female(s) per ml. Hatching was observed for up to 48 hours. Sixty egg-carrying females comprised each experimental group.

Effect of conditioned medium on hatching

To ascertain the influence of conditioned media on hatching, single females carrying mature egg-sacs were maintained in 1 ml of either conditioned or control medium. Each experimental group comprised 48 females.

Effect of killing mothers on hatching

Does inhibition of hatching by conditioned medium act directly on the embryos, or indirectly, by way of the mother? To determine this, females carrying mature egg-sacs were killed by mechanical injury to the anterior cephalothorax, taking care not to harm the egg-sac. The females were then each transferred to 1 ml of either fresh or conditioned medium. Each experimental group had 48 egg-carrying females.

Effect of detachment from mother on hatching

Mature egg-sacs were detached as one unit, without harming the mother, by a method described earlier (Provasoli *et al.*, 1959; Betouhim-El and Kahan, 1972). Each egg-sac was then placed in 1 ml of conditioned medium. Sixty-two egg-sacs were observed. The above-mentioned experiments, presented graphically, were drawn on a Macintosh computer using Cricket graph software (Figs. 2–4).

Electron microscopy

For scanning and transmission electron microscopy, mature egg sac-carrying females were fixed in a solution containing 2.5% (w/v) glutaraldehyde in 0.1 *M* Na cacodylate buffer, pH 7.4, and 2.5% NaCl. For transmission electron microscopy, females were punctured immediately after transfer to the fixative to enhance penetration, left for three hours, then washed in cacodylate buffer 0.1 *M*, left overnight, and post-fixed with 1% (w/v) osmium tetroxide for 1 h. Dehydration was through graded ethanol solutions, then embedded in Spurr's medium. Thin sections were picked up on bare copper grids and stained in 1% (w/v) lead citrate for 5 min. Sections were examined in an AEJ electron microscope at 60 kV. For scanning electron microscopy, intact females were fixed, washed, and dehydrated as described above, dried by the critical point method, coated with gold-palladium, and viewed in a Jeol 840 scanning electron microscope.

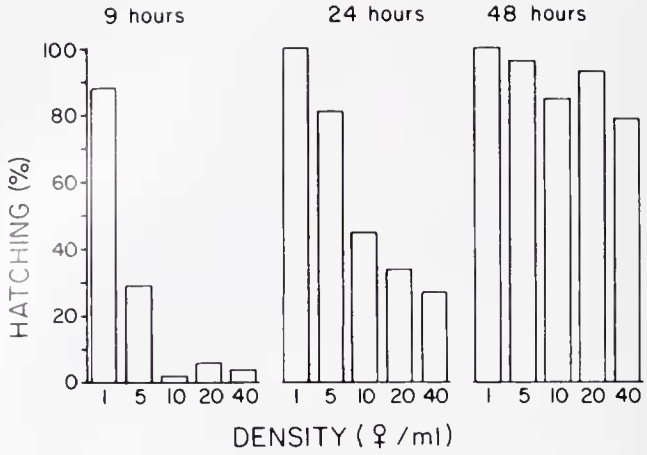

Figure 1. Effect of population density on hatching. Mature egg-sac-carrying females kept at the specified densities (1–40) in conditioned medium (see Materials and Methods). At each specified density the hatching percentage of 60 females was examined after 9, 24, and 48 hours.

Results

Effect of density on hatching

The percentage of nauplii hatching at the different experimental densities increased with time (Fig. 1). Hatching was observed in all groups after 9 hours. At the higher densities the hatching percentage was remarkably low, whereas at the lower densities (5 and 1) hatching percentage was higher (30 and 90%, respectively). After 24 hours, more mature egg-sacs had hatched in all the groups, revealing an inverse relationship between hatching percentage and population density. After 48 hours, hatching reached over 80% even at the highest density.

Effect of conditioned medium on hatching

The medium hatching time (time taken for hatching of the first 50% of the egg-sacs) in the conditioned medium was about 12 hours, whereas in the control (fresh medium) it was about 6 hours (Fig. 2).

Effect of killing mother on hatching

Figure 3 depicts the cumulative hatchings of egg-sacs remaining attached to recently killed females. Median

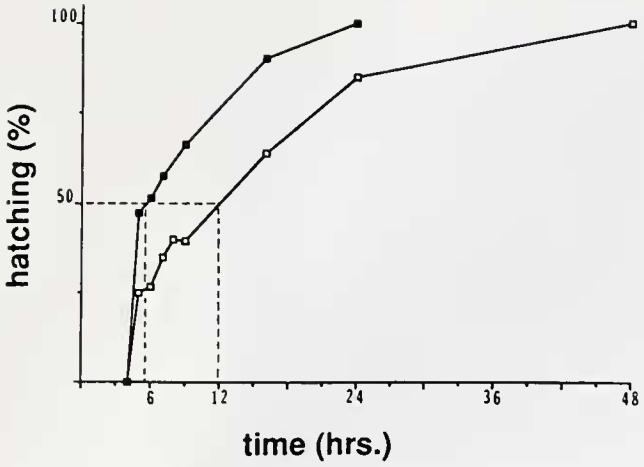

Figure 2. Effect of fresh (■ — ■) and conditioned medium (□ — □) on hatching of nauplii from mature egg-sacs carried by mothers isolated at density of 1/ml. Each experimental group consisted 48 females. Hatching percentage recorded as indicated, for a period to 48 hours. Dotted lines indicate time required for 50% of the females to hatch at each treatment.

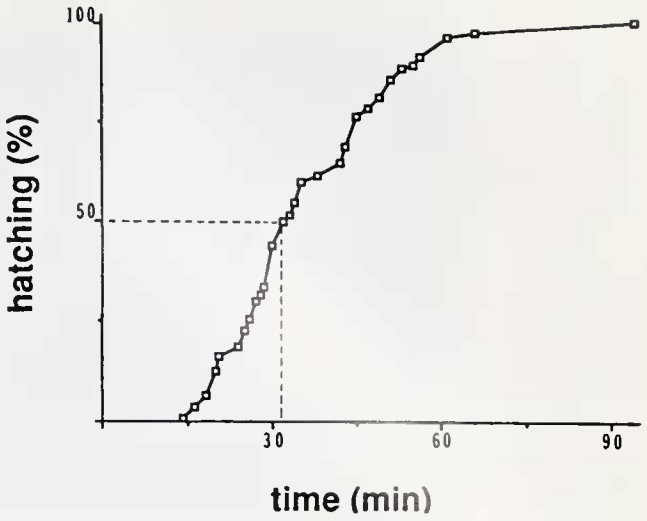

Figure 4. Hatching of nauplii from mature egg-sacs detached from the mother; 62 egg-sacs transferred. Each was placed in 1 ml conditioned medium. Dotted line indicates time for 50% of egg-sacs to hatch.

hatching time was less than a half-hour with conditioned as well as fresh medium.

Effect of detachment from mother on hatching

Figure 4 depicts the cumulative hatching of detached egg-sacs. The median hatching time resembled that for egg-sacs of killed mothers, *i.e.,* half an hour, even though the detached egg-sacs had been transferred to conditioned media.

Electron microscopy

Microscopy revealed a physical link between mother and egg-sac. Figure 5, a scanning electron micrograph, shows the ventral side of an egg-carrying female. In Figure 6, a lateral view of the abdomen of an egg-carrying female, one of the two connections are marked by an arrow. Figure 7, a scanning electron micrograph of a detached egg-sac, shows stubs of the two connections to the genitalia (arrows) on the anterior dorsal surface of the egg-sac. The transmission electron micrograph (Fig. 8) is a sagittal section through one of the two genital openings of the female, showing the connection.

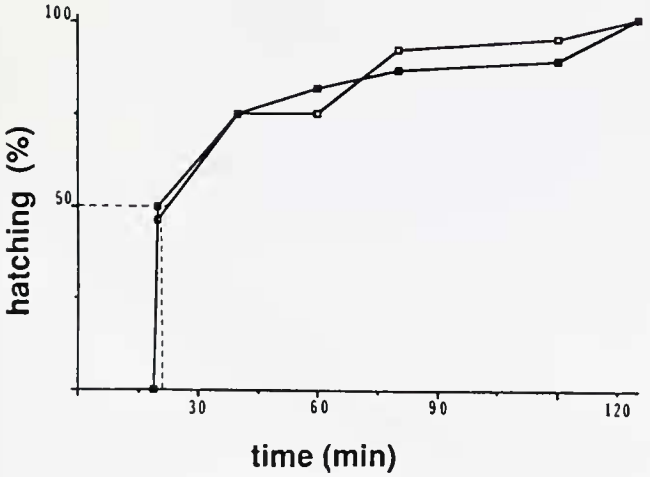

Figure 3. Effect of fresh (■ — ■) and conditioned medium (□ — □) on hatching of nauplii from mature egg-sacs carried by recently killed females; details as in Figure 2.

Discussion

The increased percentage of females carrying mature egg-sacs, first observed by us in dense cultures of *Tigriopus japonicus,* seemed to indicate a delay associated with high population density in the hatching of mature embryos. The results in Figures 1 and 2 confirm that high densities of egg-carrying females, or conditioned medium taken from dense cultures, tend to inhibit hatching, that effect diminishing considerably after 48 hours (Fig. 1).

The inhibitory effect probably is not exerted directly on the mature embryos, but rather, by the carrying mother as concluded from findings that [illegible] embryos hatched rapidly from detached egg-[illegible]en after transfer to conditioned medium. Pr[illegible] *al.* (1959) also noticed rapid hatching of detac[illegible]re egg-sacs from *T. japonicus* and *T. californic*[illegible] attributed this to nearness to an incandescent lamp. Evidence for the mother's involvement in the inhibitions was strengthened by the fact that mature egg-sacs attached to recently killed mothers hatched as rapidly as did detached egg sacs (Figs. 3, 4).

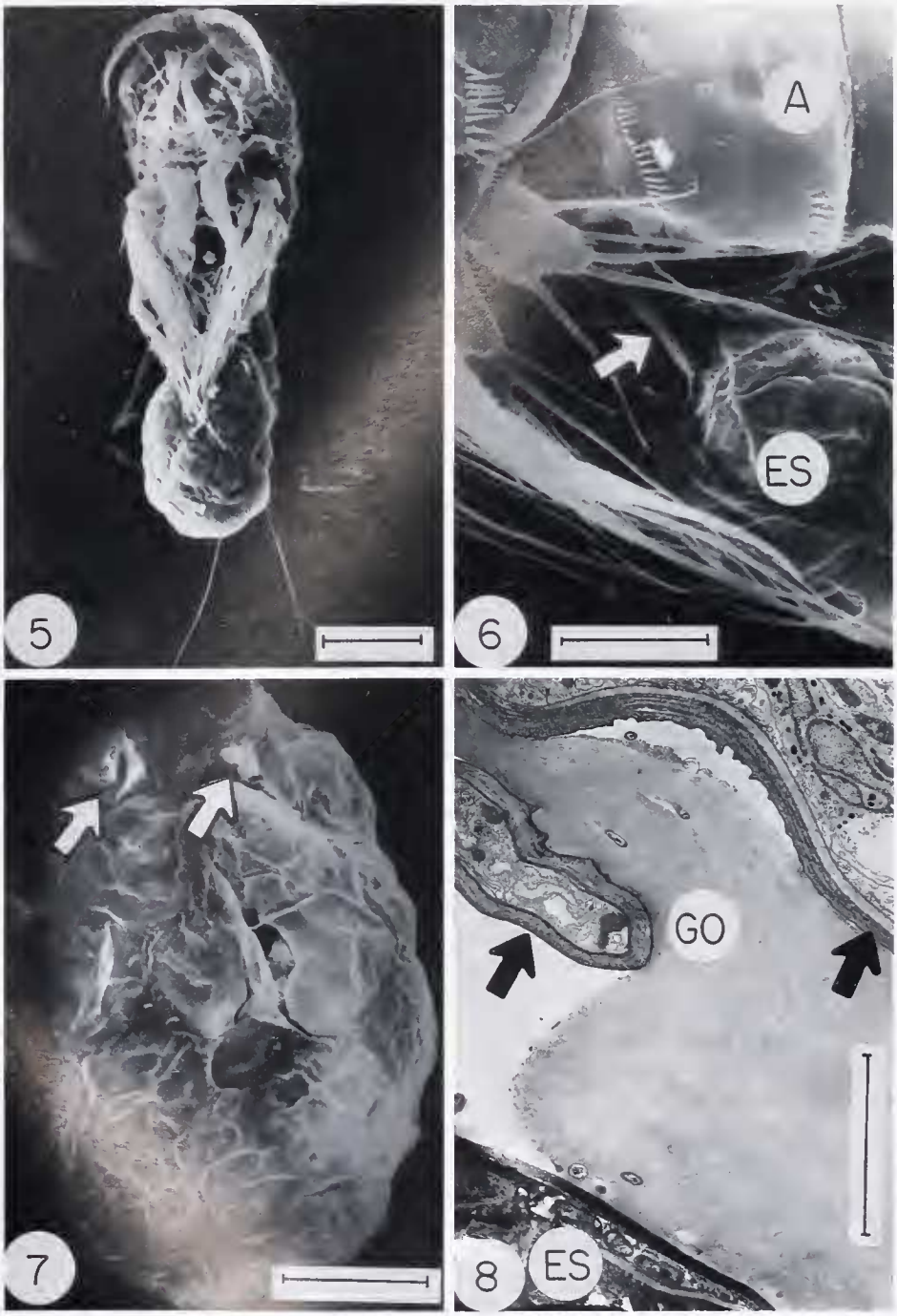

Figure 5. Scanning electron micrograph of an egg-carrying female. Ventral view. Bar: 200 μm.

Figure 6. Scanning electron micrograph of the abdomen of an egg-sac-carrying female (left lateral view); setae of appendages in foreground. Arrow indicates connection between abdomen of mother (A) and egg-sac (ES). Bar: 25 μm.

Figure 7. Scanning electron micrograph of detached egg-sac (dorsal view). Arrows indicate stubs of the two connections to the genitalia on the anterior surface. Bar: 50 μm.

Figure 8. Transmission electron micrograph of connection between genital opening (GO) and egg-sac (ES). Sagittal section; arrows indicate abdominal cuticle. Bar: 5 μm.

Injury to or removal of the head triggers egg-laying in many invertebrates, therefore attributed to annullment of a cerebrally controlled inhibition (Adiyodi and Adiyodi, 1983). But in *T. japonicus*, hatching of mature egg-sacs is evidently not triggered by their being pushed out—detached—by laying down of a new egg-sac. Females killed by mechanical injury to the cephalothorax did not lay down a new egg-sac, and the nauplii hatched while the egg-sac remained attached to the mother. Moreover, nauplii normally hatch from the egg-sac while it is carried by the female; the newly laid egg-sac appears only after the hatching of the mature one. Walker (1979) also noted that in the marine harpacticoid copepod *Amphiascoides*, a new paired sac forms only upon release of the former pair.

The live mother also mediates recovery from the inhibitory effect of crowding as inferred by comparing Figures 2 and 3, derived from experiments with copepods from the same culture and performed under identical conditions. For egg-sacs carried by a recently killed mother, the inhibition is annulled quickly. The median hatching time is within a half-hour (Fig. 3). But when they are carried by living mothers, inhibition continues for a long time, even following transfer to fresh medium. The median hatching time is about 6 hours (Fig. 2).

Inhibition depends on some sort of connection between mother and mature embryos: mere proximity of the unharmed mother to her detached egg-sac did not prevent early hatching; when only part of an egg-sac was detached, all eggs in the detached portion hatched quickly. Those remaining in the attached portion hatched much later (preliminary results). The structure shown in Figures 6–8 may serve as a conduit between the mother and her egg-sac. Accordingly, we postulate that inhibition of hatching in mature embryos is controlled by the mother through an "umbilical cord"-mechanism triggered by adverse conditions, *e.g.*, crowding. It reveals a new form of maternal care in organisms that maintain contact with their eggs until hatching. The delay of hatching of mature embryos found in *T. japonicus* may be analogous to developmental arrest in mammals (Renfree, 1978), which also maintain contact with their developing embryos.

Another type of maternal involvement occurs in other crustaceans *e.g.*, notodelphyoid copepods (Davis, 1968) and the crab *Rhithropanopeus harrisii* (Forward and Lohmann, 1983). In the copepods the mother's movements help nauplii to emerge; in the crab, a chemical cue released by the first larvae triggers the mother's aid in the hatching of the remaining brood. In cirripeds, a small amount of the mother's hemolymph promotes hatching of mature eggs (Crisp, 1956, 1969; Crisp and Spencer, 1958). Enhanced hatching in *Tigriopus* (Figs. 3, 4) may be triggered by a substance released from an unnoticed wound inflicted on the mother while the egg-sac was detaching. This possibility was eliminated, as no enhanced hatching was noticed, in a preliminary experiment wherein homogenates of *Tigriopus* mothers were added to the medium where mature egg sac-carrying females were maintained. Maternal control of hatching in the aforementioned various crustaceans is thus promotive. It is inhibitory in *Tigriopus*.

A reproductive strategy involving delay in the hatching of nearly mature embryos from eggs laid and no longer in contact with the mother occurs in the squid *Loligo vulgaris* (Marthy *et al.*, 1976; Weischer and Marthy, 1983). Delay is caused by a tranquilizer in the perivitelline fluid of the eggs. However, a natural tranquilizer seems uninvolved in inhibition of hatching in *T. japonicus* since a macerate of mature egg-carrying females (40 per ml) did not tranquilize nauplii.

The maternally inhibited hatching mechanism described here might be advantageous for *T. japonicus* in its tide-pool habitat, which is characterized by diurnal short-period fluctuations. Igarashi (1959) did find wide fluctuations in population density and age composition of *T. japonicus* in various kinds of tidal pools. When tidal pools are densely populated, the inhibitory mechanism could delay hatching of offspring until the next tide distributed the nauplii to less crowded habitats. The inhibitory period of up to 48 hours found in the highest densities of females tested (Fig. 1) could also be advantageous for populations in tidal pools where fluctuations are less frequent *e.g.*, pools located at higher tidal levels. This delayed mechanism alone, or along with others found in various copepods under crowded culture conditions [*e.g.*, changes in age distribution, sex ratio, fertile period, number of ovisacs and eggs (Hicks and Coull 1983; Walker, 1979; Kahan and Azoury 1981; Kahan *et al.*, unpubl.)] might regulate population growth in *T. japonicus*. Whether the regulation in *Tigriopus* is common in other copepods and aquatic organisms remains to be determined. A better understanding of hatching in aquatic invertebrates, urged by Davis (1981), should help elucidate the mechanism of maternally controlled hatching in *T. japonicus*.

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